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(54) Title: METHODS OF STIMULATING ANGIOGENESIS

(57) Abstract

The invention is directed to methods for stimulating angiogenesis by *in vivo* intramuscular, intradermal, and/or subcutaneous administration of cationic lipid–nucleic acid complexes. By inducing angiogenesis, these compositions are used to treat ischemia, including diseases which cause or result in insufficient circulation to and perfusion of tissues, such as peripheral vascular disease (e.g., as in diabetes, atherosclerosis) and coronary artery disease.

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METHODS OF STIMULATING ANGIOGENESIS

FIELD OF THE INVENTION

This invention pertains generally to the induction and stimulation of angiogenesis *in vivo* by intramuscular, intradermal, and subcutaneous administration of cationic lipid - nucleic acid complexes. In particular, this invention pertains to the treatment of diseases which cause or result in ischemia, such as peripheral vascular disease and coronary artery disease.

INTRODUCTION

Diseases and conditions causing or involving tissue ischemia are major health concerns. Ischemia is seen, for example, in coronary artery disease (CAD) and peripheral vascular disease (PVD). It has been reported by the American Heart Association that there are about 60 million adults in the United States with cardiovascular disease, including 11 million adults with coronary heart disease. Angina, a symptom of heart ischemia, afflicts 1.5 million adults in the United States, with about 350,000 new cases a year. It is estimated that PVD affects 30 percent of the adult population. A primary cause of PVD, atherosclerotic vascular disease, coronary heart disease (CHD), and cerebrovascular disease is diabetes mellitus.

Ischemia occurs when a tissue receives an inadequate supply of blood. For example, myocardial ischemia occurs when cardiac muscle does not receive an adequate blood supply. This can be due to occlusion or narrowing of the blood vessels, such as seen in coronary artery atherosclerosis. Treatments include surgical and pharmaceutical approaches. Surgical intervention is used to widen the narrowed lumens (*e.g.*, balloon angioplasty) or to increase the numbers of cardiac blood vessels (*e.g.*, bypass surgery using grafts). Less traumatic pharmaceutical treatments act to decrease cardiac muscle demand for oxygen and nutrients or to increase the blood supply. Oxygen demand can be lowered by decreasing the contractile response of the heart to a hemodynamic load (*e.g.*, using beta-adrenergic blockers). Cardiac blood

supply can be augmented by increasing the diameter of smooth muscle-walled coronary artery vessel lumens (as with nitroglycerin or calcium channel blockers). However, these pharmaceutical treatments are inexact, transiently active, and highly prone to drug interactions and side effects.

Another means to increase blood supply to an ischemic tissue is to induce the growth of blood vessels to the tissue (angiogenesis) or to increase the amount of blood bathing the tissues (increased blood perfusion). This has been accomplished by administration of angiogenic growth factors. Several angiogenic proteins have been identified, including, for example, vascular endothelial growth factors (VEGFs). VEGFs are angiogenic and permeability-inducing factors. VEGFs are important mediators of angiogenesis, as they act directly and specifically on endothelial cells. Grad (1998) Clin. Chem Lab Med. 36:379-383. *In vivo*, they are associated with blood vessel growth in development, wound repair (angiogenesis is a key component of the repair mechanisms triggered by tissue injury), cancer, and other diseases and conditions.

To achieve an angiogenic effect, repeated and/or long term administration of a polypeptide blood vessel growth factor, such as VEGF, would be needed. This approach, however, is typically very costly and inconvenient, as it usually requires repeated administrations by injection.

Alternatively, a polypeptide blood vessel growth factor can be administered *in vivo* by delivering not the polypeptide itself, but instead, the nucleic acid which encodes it. Angiogenic genes have been administered *in vivo* intravascularly. See., *e.g.*, Laitinen (1998) Hum. Gene Ther. 9:1481-1486; Isner (1997) Adv. Drug Deliv. Reviews 30:185-197; Giordano (1996) Nature Med. 2:534-539; Takeshita (1996) Lab. Invest. 75:487-501; Mc Donald, *et al.*, U.S. Patent No. 5,837,283 (" '283").

A novel means to stimulate angiogenesis, described for the first time in the present invention, involves administration of angiogenic factors intramuscularly (IM) and subcutaneously (SC). While polypeptide-encoding genes have been injected intramuscularly (as naked plasmid DNA or viral expression vectors), to date, no angiogenic factors have been administered directly into the muscle (IM) or into the skin (SC); see, *e.g.*, Baumgartner (1998) Circulation 97:1114-1123; Tsurumi (1997)

Circulation 96(9 Suppl):II-II3828; Hammond, et al., U.S. Patent No. 5,792,453; and Mc Donald, *et al.*, '283.

Considering the increasing numbers of individuals in our aging population afflicted with disease and conditions involving ischemic tissues, new treatments for ischemia that are safer and more predictable are needed. The present invention provides these needs and related advantages.

SUMMARY OF THE INVENTION

The invention provides methods for stimulating angiogenic activity in a tissue by intramuscular, intradermal, or subcutaneous administration of a pharmaceutical composition. The pharmaceutical composition comprises a pharmaceutically acceptable carrier and a cationic lipid - nucleic acid complex. The pharmaceutical composition is administered in an amount effective to induce angiogenic activity (defined below) in the tissue. In alternative embodiments, the pharmaceutical composition is administered into a skeletal muscle or a cardiac muscle; or, the subcutaneous/ intradermal administration is an intradermal injection.

In one embodiment, the pharmaceutical composition is administered in a unit dosage form. The unit dosage form can deliver between about 1 ngm to about 6 ngm, or, alternatively, about 2 ngm, of the nucleic acid-cationic lipid complex. In another embodiment, the unit dosage form of about 2 ngm of nucleic acid is administered in at least two intervals about 4 weeks apart. In alternative embodiments, the pharmaceutical composition is in the form of an injectable solution and the pharmaceutically acceptable carrier is an aqueous solution.

In one embodiment, the cationic lipid complex can have a net positive charge, and the cationic lipid can be BODAI, DOTMA, DMRIE, DOTAP, DOGS, EDMPC, MeBOP, or DCChol. The lipid content of the cationic lipid-DNA complex formulation can be about 1.5 mM BODAI and about 1.5 mM DOPE.

In other embodiments, the nucleic acid of the cationic lipid-nucleic acid complex is DNA. The DNA can comprise a sequence that does not encode a polypeptide with biologic activity, or, it can comprise a sequence that does not encode a

polypeptide with angiogenic activity. Alternatively, the DNA can comprises a sequence that encodes a polypeptide with biologic activity, including, for example, a polypeptide having an angiogenic activity, such as a vascular endothelial growth factor activity. In one embodiment, the DNA comprises a sequence encoding a vascular endothelial growth factor polypeptide, such as, for example, the polypeptide having a sequence as set forth in SEQ ID NO:2.

The present invention also provides a method for treating ischemia in a tissue, the method comprising intramuscular, intradermal, or subcutaneous administration of a pharmaceutical composition comprising a pharmaceutically acceptable carrier and a cationic lipid -nucleic acid complex in an amount effective to treat the ischemia in the tissue. In alternative embodiments, the ischemia is caused by peripheral vascular disease, such as, for example, diabetes, atherosclerosis, coronary artery disease, and the like.

A further understanding of the nature and advantages of the present invention may be realized by reference to the remaining portions of the specification, the figures and claims.

All publications, patents and patent applications, including GenBank and ATCC library database references, as cited herein, are hereby expressly incorporated by reference for all purposes to the same extent as if fully set forth herein.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the ratios of blood flow between ischemic and normal perfused gastrocnemius muscles measured at different times after surgical induction of ischemia and IM administration of cationic lipid-nucleic acid compositions of the invention at different concentrations, as explained in Example 1. Statistically significant differences between treatment groups and control are observed on weeks one and two after surgery.

Figure 2 shows a plot schematically summarizing the dynamics and the rate of restoration of the blood flow in ischemic gastrocnemius muscle after IM administration of angiogenic cationic lipid-nucleic acid compositions, as explained in Example 1.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to pharmaceutical compositions and methods for stimulating angiogenesis *in vivo*. by intramuscular (IM) intradermal, and subcutaneous (SC) administration. The compositions comprise cationic lipid - nucleic acid complexes, where the nucleic acid may or may not be the same or complementary to a naturally transcribed sequence. In one embodiment, the nucleic acid is DNA encoding a polypeptide with angiogenic activity. By inducing angiogenesis, these compositions are used to treatment ischemia, including diseases which cause or result in insufficient circulation to and perfusion of tissues, such as peripheral vascular disease (*e.g.*, as in diabetes, atherosclerosis) and coronary artery disease.

The invention is also directed to methods of stimulating angiogenesis, thereby treating ischemia, using intramuscularly, intradermally, and subcutaneously administered pharmaceutical compositions comprising cationic lipid - nucleic acid complexes. This aspect of the invention is based on the surprising discovery that angiogenesis can be induced by intramuscular (IM), intradermal, and/or subcutaneous (SQ) administration of a complex comprising cationic lipid and any nucleic acid. That is, cationic lipid-nucleic acid complexes possess an inherent ability to stimulate angiogenesis, without regard to a specific gene product, if any, encoded by the nucleic acid, when injected IM or SQ. In one embodiment, the nucleic acid is DNA. In a preferred embodiment, the DNA encodes a polypeptide which itself has angiogenic activity, thus imbuing an additive or synergistic angiogenic effect.

Definitions

To facilitate understanding the invention, a number of terms are defined below.

As used herein, the term "angiogenesis" refers to stimulation or induction of an increased rate of, or *de novo* formation of, blood vessels. *e.g.*, capillaries, see *e.g.*, Folkman (1992) Nature Med. 1:27-21. Compositions can be screened for angiogenic activity *in vitro* or *in vivo*. An exemplary *in vitro* capillary formation assessment uses endothelial cells imbedded in Matrigel matrix (Collaborative Research, Bedford, MA), as

described by, *e.g.*, Deramaudt (1998) J. Cell. Biochem. 68:121-127). *In vivo* animal models are discussed below.

As used herein, the term "cationic lipid - nucleic acid complex" refers to a non-covalent association between a cationic lipid moiety and a nucleic acid. Typically, the positively charged lipid will associate with the negatively charged nucleic acid by charge interactions. The complex can include any number of additional constituents, such as, *e.g.*, neutral lipids, as discussed below. Means of making these complexes are discussed below.

The term "expression cassette" refers to any recombinant expression system for the purpose of expressing a nucleic acid sequence of the invention *in vitro* or *in vivo*, constitutively or inducibly, in any cell. The term includes linear or circular expression systems. The term includes, *e.g.*, vectors, that remain episomal or integrate into the host cell genome. The expression cassettes can have the ability to self-replicate or not, *i.e.*, drive only transient expression in a cell. The term includes recombinant expression cassettes which contain only the minimum elements needed for transcription of the recombinant nucleic acid, such as one which encodes a polypeptide with angiogenic activity. In one embodiment, the cationic lipid- nucleic acid complex of the invention comprises a DNA encoding a polypeptide with angiogenic activity in the form of an expression cassette. In an alternative embodiment, the nucleic acid may or may not be the same or complementary to a naturally transcribed sequence. Thus, the DNA insert in the expression cassette may be non-coding, *i.e.*, an "empty vector."

As used herein, the term "wherein the pharmaceutical composition is administered intramuscularly or subcutaneously" incorporates the common usage of intramuscular (IM) and subcutaneous (SC), and includes any means of IM or SC administration known in the art, including, *e.g.*, all forms of skeletal, smooth or cardiac muscle injections and subcutaneous or intradermal injections (see, *e.g.*, Fjellner (1983) *Acta Derm Venereol* 63:456-457; Ross (1997) *Clin Cancer Res* 3:2191-2196; Ciernik (1996) *Hum Gene Ther* 7:893-899; Eriksson (1998) *J Surg Res* 78:85-91).

The term "lipid" as used herein incorporates its common usage; and includes neutral, anionic, cationic and amphiphilic compositions, both naturally occurring and synthetic. The term "cationic lipid" includes any lipid with a net positive charge at neutral pH, such as under physiologic conditions. A cationic lipid can be a positively

charged lipid comprising, *e.g.*, a quaternary ammonium salt moiety. Cationic lipids can consist of a hydrophilic polar head group and lipophilic aliphatic chains. Similarly, cholesterol derivatives having a cationic polar head group can also be used, see, *e.g.*, Farhood (1992) Biochim. Biophys. Acta 1111:239-246. The cationic lipid may be used in combination with other cationic lipids, or with neutral or anionic lipids. The cationic lipid may be in any physical form including, *e.g.*, liposomes, micelles, interleaved bilayers, and the like. Cationic lipids are described in further detail, below.

As used herein, the term "nucleic acid" or "nucleic acid sequence" refers to any deoxyribonucleotide or ribonucleotide sequence in, *e.g.*, single-stranded, double-stranded or triplex form. The term encompasses nucleic acids, *e.g.*, oligonucleotides, containing known naturally occurring nucleotides, analogues of natural nucleotides, and mixtures thereof. The term also encompasses nucleic-acid-like structures with synthetic backbones. DNA backbone analogues provided by the invention include phosphodiester, phosphorothioate, phosphorodithioate, methyl-phosphonate, phosphor-amidate, alkyl phosphotriester, sulfamate, 3'-thioacetal, methylene (methylimino), 3'-N-carbamate, morpholino carbamate, and peptide nucleic acids (PNAs); see Oligonucleotides and Analogues, a Practical Approach, edited by F. Eckstein, IRL Press at Oxford University Press (1991); Antisense Strategies, Annals of the New York Academy of Sciences, Volume 600, Eds. Baserga and Denhardt (NYAS 1992); Milligan (1993) J. Med. Chem. 36:1923-1937; Antisense Research and Applications (1993, CRC Press). PNAs contain non-ionic backbones, such as N-(2-aminoethyl) glycine units. Phosphorothioate linkages are described in WO 97/03211; WO 96/39154; Mata (1997) Toxicol. Appl. Pharmacol. 144:189-197. Other synthetic backbones encompasses by the term include methyl-phosphonate linkages or alternating methylphosphonate and phosphodiester linkages (Strauss-Soukup (1997) Biochemistry 36:8692-8698), and benzylphosphonate linkages (Samstag (1996) Antisense Nucleic Acid Drug Dev. 6:153-156). As described above, the angiogenic action of the nucleic acid-cationic lipid complex is independent of whether the nucleic acid encodes any transcribable sequence, such as a naturally occurring polypeptide. In one embodiment, the nucleic acid is DNA. In a preferred embodiment, the DNA

encodes a polypeptide which itself has angiogenic activity; and, can be in the form of an expression cassette, as discussed above.

The terms "ischemia," "peripheral vascular disease," "diabetes," "atherosclerosis," and "coronary artery disease" as used herein, incorporates their common usages.

As used herein, the term "pharmaceutically acceptable carrier" includes any suitable pharmaceutical excipient, including, *e.g.*, water, saline, phosphate buffered saline, Hank's solution, Ringer's solution, dextrose/saline, glucose, lactose, or sucrose solutions, starch, cellulose, talc, gelatin, malt, rice, flour, chalk, silica gel, magnesium stearate, sodium stearate, glycerol monostearate, dried skim milk, glycerol, propylene glycol, ethanol, and the like.

As used herein, the term "polysaccharide" or "oligosaccharide" incorporates its common usages, and includes, *e.g.*, dextrose, glucose, lactose, mannose, mannan, and the like, as described below.

As used herein, the term "wherein the DNA comprises a sequence that is not the same or complementary to a naturally transcribed DNA sequence" means that the DNA sequence cannot generate a peptide or polypeptide which is normally or naturally produced by the tissue into which it is administered. For example, the DNA sequence is not the same or complementary to a naturally transcribed DNA sequence if, when administered to a human as part of the nucleic acid-cationic lipid pharmaceutical of the invention, it cannot generate a peptide or polypeptide naturally produced by the human. For example, a reporter gene or a marker gene (*e.g.*, for an epitope tag) would not be a naturally transcribed DNA sequence .

As used herein, the term "wherein the DNA comprises a sequence that does not encode a polypeptide with biologic activity" means the polypeptide encoded by the DNA sequence, when expressed in an animal, does not have any effect on the normal cell biology or physiology of the organisms. For example, cell growth, differentiation, apoptosis, and the like are biological activities, including, *e.g.*, "vascular endothelial growth factor activity," "angiogenic activity," and "angiogenic growth factor activity" (described below). In contrast, the ability to act an as antigen, a reporter gene, a marker (*e.g.*, an epitope tag) would not be considered a biological activity.

As used herein, the term "wherein the DNA comprises a sequence that does not encode a polypeptide with angiogeneic activity" means the polypeptide encoded by the DNA sequence, when expressed in an animal, does not have any effect on "vascular endothelial growth factor activity," "angiogenic activity," and "angiogenic growth factor activity" as described below.

As used herein, the terms "vascular endothelial growth factor activity," "angiogenic activity," and "angiogenic growth factor activity" include a broad range of physiologic activities that increase the amount of blood flow to a tissue, including, *e.g.*, increased vascular permeability, increased vascular density, endothelial cell (EC) activation, EC migration, EC proliferation, capillary formation (angiogenesis), vasculogenesis (the *de novo* organization of ECs into vascular structures) and neovascularization (see, *e.g.*, Folkman (1992) *supra*). Angiogenic activity may include, *e.g.*, growth factors that induce angiogenesis, or inhibitors of angiogenesis inhibitors, or factors which induce expression of endogenous growth factors (*e.g.*, gene activators or transcriptional regulators). The terms "vascular endothelial growth factor" or "VEGF" includes growth factors which, alone or in combination with other growth factors, such as fibroblast growth factor, can initiate vascular development, angiogenesis and other angiogenic activities. VEGFs include the family of VEGF genes and their three alternatively spliced forms, including VEGF (VEGF-A); VEGF-B; VEGF-C (or VEGF-2); $VEGF_{115}$; $VEGF_{145}$; $VEGF_{121}$; $VEGF_{165}$; $VEGF_{189}$; and $VEGF_{256}$; see, *e.g.*, Olofsson (1996) J. Biol .Chem. 271:19310-19317; Olofsson (1996) Proc. Natl. Acad. Sci. USA 93:2576-2581; Sugihara (1998) J. Biol. Chem. 273:3033-3038; Poltorak (1996) J. Biol. Chem. 272:7151; Beck (1997) FASEB J. 11:365-373; Witzenbichler (1998) Am. J. Pathol. 153:381-394). A variety of *in vivo* animal models can be used to evaluate the ability of a cationic lipid-nucleic acid complex of the invention to have angiogenic activity (in addition to the *in vitro* test described above, see Folkman (1992) *supra*). For example, neovascularization of ischemic muscle can be demonstrated by experiments in which exogenously administered cationic lipid-nucleic acid complexes of the invention augment collateral blood flow in experimentally induced mouse or rabbit hindlimb ischemia; see, *e.g.*, Couffinhal (1998) Am. J. Pathol. 152:1667-1679; Witzenbichler (1998) *supra*.

Pharmaceutical Compositions for Inducing Angiogenesis

This invention provides methods for inducing angiogenesis by intramuscular, intradermal, and/or subcutaneous administration of a pharmaceutical composition. The composition comprises a pharmaceutically acceptable carrier and a pharmacologically effective amount of a cationic lipid-nucleic acid complex. In one embodiment, the nucleic acid is a DNA comprising no sequences capable of generating a polypeptide, naturally occurring or otherwise. In an alternative embodiment, the nucleic acid of the complex encodes an angiogenic growth factor polypeptide.

The invention, for the first time, provides methods of using these pharmaceutical compositions to induce angiogenic activity and to treat ischemia by intramuscular (IM), intradermal, or subcutaneous (SC) administration. It is an advantage of the present invention that high levels of angiogenesis can be stimulated by IM, intradermal, or SC administration of cationic lipid - nucleic acid complexes. This is a much simpler and safer mode of administration than intravascular (IV) delivery. IM, intradermal, and SC modes of delivery are less invasive than IV. They are also more amenable to repeat administrations.

The invention can be practiced in conjunction with any appropriate method or protocol known in the art, which are well described in the scientific and patent literature. Therefore, only a few general techniques will be described prior to discussing specific methodologies and examples relative to the novel reagents and methods of the invention.

General Techniques

The nucleic acids used in the cationic-lipid complexes of this invention, whether RNA, cDNA, genomic DNA, oligonucleotides, or hybrids thereof, may be isolated from a variety of sources, genetically engineered, amplified, and/or expressed recombinantly. Any recombinant expression system can be used, including, *e.g.*, bacterial, yeast, insect or mammalian systems. Alternatively, these nucleic acids can be chemically synthesized *in vitro* (see definition of nucleic acids). Techniques for the manipulation of nucleic acids, such as, *e.g.*, subcloning into expression vectors, labeling probes, sequencing DNA, DNA hybridization are described in the scientific and patent literature, see *e.g.*, Sambrook, ed., MOLECULAR CLONING: A LABORATORY MANUAL (2ND ED.), Vols. 1-3, Cold Spring Harbor Laboratory, (1989) ("Sambrook"); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Ausubel, ed. John Wiley & Sons, Inc., New York (1997)

("Ausubel"); and, LABORATORY TECHNIQUES IN BIOCHEMISTRY AND MOLECULAR BIOLOGY: HYBRIDIZATION WITH NUCLEIC ACID PROBES, Part I. Theory and Nucleic Acid Preparation, Tijssen, ed. Elsevier, N.Y. (1993) ("Tijssen"). Product information from manufacturers of biological reagents and experimental equipment also provide information regarding known biological methods. Such manufacturers include the SIGMA chemical company (Saint Louis, MO), R&D systems (Minneapolis, MN), Pharmacia Biotech (Piscataway, NJ), Clontech Laboratories, Inc. (Palo Alto, CA), Aldrich Chemical Company (Milwaukee, WI), GIBCO BRL Life Technologies, Inc. (Gaithersburg, MD), Fluka Chemica-Biochemika Analytika (Fluka Chemie AG, Buchs, Switzerland), Applied Biosystems (Foster City, CA), as well as many other commercial sources known to one of skill.

The nucleic acids of the invention can also be generated or quantitated using amplification techniques. Suitable amplification methods include, but are not limited to: polymerase chain reaction, PCR (PCR PROTOCOLS, A GUIDE TO METHODS AND APPLICATIONS, ed. Innis, Academic Press, N.Y. (1990) and PCR STRATEGIES (1995), ed. Innis, Academic Press, Inc., N.Y. (Innis)), ligase chain reaction (LCR) (Wu (1989) Genomics 4:560; Landegren (1988) Science 241:1077; Barringer (1990) Gene 89:117); transcription amplification (Kwoh (1989) Proc. Natl. Acad. Sci. USA, 86:1173); and, self-sustained sequence replication (Guatelli (1990) Proc. Natl. Acad. Sci. USA, 87:1874); Q Beta replicase amplification (Smith (1997) J. Clin. Microbiol. 35:1477-1491, automated Q-beta replicase amplification assay; Burg (1996) Mol. Cell. Probes 10:257-271) and other RNA polymerase mediated techniques (*e.g.*, NASBA, Cangene, Mississauga, Ontario); see also Berger (1987) Methods Enzymol. 152:307-316, Sambrook, Ausubel, Mullis (1987) U.S. Patent Nos. 4,683,195, and 4,683,202; Arnheim (1990) C&EN 36-47; Lomell J. Clin. Chem., 35:1826 (1989); Van Brunt (1990) Biotechnology, 8:291-294; Wu (1989) Gene 4:560; Sooknanan (1995) Biotechnology 13:563-564. Methods for cloning *in vitro* amplified nucleic acids are described in Wallace, U.S. Pat. No. 5,426,039.

Nucleic acids and lipids are analyzed and quantified by any of a number of general means well known to those of skill in the art. These include, *e.g.*, analytical biochemical methods such as NMR, spectrophotometry, radiography, electrophoresis,

capillary electrophoresis, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), and hyperdiffusion chromatography, various immunological methods, such as fluid or gel precipitin reactions, immunodiffusion (single or double), immunoelectrophoresis, radioimmunoassays (RIAs), enzyme-linked immunosorbent assays (ELISAs), immuno-fluorescent assays, Southern analysis, Northern analysis, dot-blot analysis, gel electrophoresis (*e.g.*, SDS-PAGE), RT-PCR, quantitative PCR, other nucleic acid or target or signal amplification methods, radiolabeling, scintillation counting, and affinity chromatography.

Nucleic Acids Encoding Angiogenic Polypeptides

In one embodiment, the invention is directed to pharmaceutical compositions of cationic lipid - nucleic acids and methods of inducing angiogenesis and treating ischemia by administering these compositions IM, intradermally, or SC, where the nucleic acid encodes no polypeptide, or, encodes a polypeptide which itself has angiogenic activity. Because the cationic lipid - nucleic acid complex alone induces angiogenic activity, *in vivo* synthesis of an angiogenic polypeptide only generates an additive or synergistic effect.

Any single or combination of nucleic acids encoding angiogenic activity-inducing polypeptides can be incorporated into the lipid-nucleic acid complex of the invention. In one embodiment, the nucleic acid encodes a vascular endothelial growth factor (VEGF) which, alone or in combination with other exogenously or endogenously derived growth factors, such as fibroblast growth factor, can initiate vascular development, increased vascular permeability (VEGF is also called "vascular permeability factor," see, *e.g.*, Hippenstiel (1998) Am. J. Physiol. 274: L678-L684), endothelial cell (EC) activation, EC migration, EC proliferation, capillary formation (angiogenesis), vasculogenesis, and neovascularization. VEGF includes several isoforms (including alternatively spliced forms): VEGF (VEGF-A), VEGF-B, VEGF-C (or VEGF-2) and VEGF-D (see, *e.g.*, Beck (1997) FASEB J. 11:365-373). When expressed *in vivo* (or *in vitro* models, as described above), VEGF promotes angiogenesis (see, *e.g.*, Witzenbichler (1998) *supra*). The role of VEGF in therapeutic angiogenesis has been demonstrated by experiments in which exogenously administered VEGF augments collateral blood flow in animals and patients with experimentally induced hindlimb or myocardial ischemia.

Human VEGF nucleic acid coding sequences and mRNA have been described, *e.g.*, by Herold (1997) Cardiovasc. Pathobiol. 2 :88-96 (see GenBank Accession No. AF024710); Matsuda, et al., Japanese patent JP 1997173075-A 1, 08-JUL-1997 (see GenBank Accession No. E13332); Achen (1998) Proc. Natl. Acad. Sci. USA 95:548-553 (see GenBank Accession No. AJ000185); Olofsson (1996) J. Biol. Chem. 271:19310-19317 (see GenBank Accession No. U52819); Leung (1989) Science 246:1306-1309 (see GenBank Accession No. M32977); Tischer, et. al., United States Patent No. (USPN) 5,219,739; Robinson, G.S., USPN 5,661,135; Chen, et. al., USPN 5,073,492.

Other angiogenic protein-encoding nucleic acids can be used in the cationic lipid-nucleic acid complexes of the invention, including, *e.g.*, any member of the family of fibroblast growth factors (FGFs); see, *e.g.*, Chen (1997) Proc. Assoc. Am. Physicians 109:351-361; Harada, et al., (1994) J. Clin. Invest. 94:623-630; Thomas, et. al., U.S. Patent No. 5,409,897; Slavin (1995) Cell Biol. Intl. 19:431-444. Other angiogenic protein-encoding nucleic acids that can be used also include angiopoietin-1, angiopoietin-2, del-1, monocyte chemotactic protein-1, see, *e.g.*, Maisonpierre (1997) Science 277:55-60; Koblizek (1998) Curr. Biol. 8:529-532.

In one embodiment of the invention, nucleic acids encoding polypeptides (including, *e.g.*, angiogenic activity-inducing polypeptides, gene activation peptides that upregulate VEGF, VEGF-R, etc., such as *trans*-acting transcriptional activators, *e.g.*, zinc finger proteins that upregulate VEGF) are used; and in an alternative embodiment, they are incorporated in expression cassettes, as described above. In these embodiments, the polypeptide's coding sequence is operably linked to a promoter. This complex can also be incorporated into a expression vector or plasmid or the like; see, *e.g.*, Hammond, et al., USPN 5,792,453, which incorporates transgenes encoding angiogenic proteins into recombinant adenovirus vectors for *in vivo* administration to induce angiogenesis.

The promoters and vectors used in this invention can be isolated from natural sources, obtained from such sources as ATCC or GenBank libraries, or prepared by synthetic or recombinant methods, as described herein. Typical expression systems contain, in addition to coding sequence, transcription and translation terminators,

polyadenylation sequences, transcription and translation initiation sequences, and transcriptional regulatory elements, *e.g.*, promoters and enhancers. The expression systems optionally contain at least one independent terminator sequence, sequences permitting replication of the cassette both *in vitro* and *in vivo*, *e.g.*, eukaryotes or prokaryotes, or a combination thereof, (*e.g.*, shuttle vectors). They may further include selection markers for the selected expression system, *e.g.*, prokaryotic or eukaryotic systems.

Cationic Lipid - Nucleic Acid Complexes

The invention provides methods for inducing angiogenesis comprising administration IM, intradermally, or SC of a cationic lipid - nucleic acid complex. Cationic lipids have been shown to mediate intracellular delivery of plasmid DNA (Felgner (1987) Proc. Natl. Acad. Sci. USA 84:7413-7416), and mRNA (Malone (1989) Proc. Natl. Acad. Sci. USA 86:6077-6081). Use of lipids complexed with nucleic acids, such as DNA, is described, *e.g.*, by Zhu (1993) Science 261:209-211; Vigneron (1996) Proc. Natl. Acad. Sci. USA 93:9682-9686; Hofland (1996) Proc. Natl. Acad. Sci. USA 93:7305-7309; Alton (1993) Nat. Genet. 5:135-142; von der Leyen (1995) Proc. Natl. Acad. Sci. USA 92:1137-1141. For a review of liposomes in gene therapy, see Lasic and Templeton (1996) Adv. Drug Deliv. Rev. 20:221-266.

Cationic Lipids

Cationic lipid carriers contain a positively charged lipid. The liposomes may have a single lipid bilayer (unilamellar) or more than one bilayer (multilamellar). They are generally categorized according to size, where those having diameters up to about 50 to 80 nm are termed "small" and those greater than about 80 to 1000 nm, or larger, are termed "large." Thus liposomes are typically referred to as large unilamellar vesicles (LUVs), multilamellar vesicles (MLVs) or small unilamellar vesicles (SUVs). Methods of producing cationic liposomes are known in the art. See, *e.g.*, Liposome Technology (CFC Press, NY 1984); Liposomes, Ortro (Marcel Schher, 1987); Methods Biochem Anal. 33:337462 (1988).

Cationic lipids that can be incorporated in the cationic lipid-nucleic acid complexes of the invention include, *e.g.*, imidazolinium derivatives (WO 95/14380), guanidine derivatives (WO 95/14381), phosphatidyl choline derivatives (WO 95/35301), and piperazine derivatives (WO 95/14651). Additional examples of cationic lipids that

may be used in the present invention include DOTIM (also called BODAI) (see, *e.g.*, Solodin (1995) Biochem. 34:13537-13544); DDAB (see, *e.g.*, Rose (1991) BioTechniques 10:520-525); DOTMA (also called N-[1-(2,3-dioleoyloxy)propyl-N,N, N-trimethylammonium chloride, see, *e.g.*, Song (1998) Biochim. Biophys. Acta 1372:141-150, and U.S. Patent No. 5,550,289); DOTAP (see, *e.g.*, Eibl and Wooley (1979) Biophys. Chem. 10:261-271); DMRIE (see, *e.g.*, Felgner (1994) J. Biol. Chem. 269:2550-2561; Huang (1998) Chem. Biol. 5:345-354); EDMPC (see, *e.g.*, Avanti Polar Lipids, Alabaster, AL); DCChol (see, *e.g.*, Gau and Huang (1991) Biochem. Biophys. Res. Comm. 179:280-285); DOGS (see, *e.g.*, Behr (1989) Proc. Natl. Acad. Sci. USA 86:6982-6986; Meyer (1998) J. Biol. Chem. 273:15621-15627); MBOP (also called MeBOP) (see, *e.g.*, WO 95/14651); and, those described in WO 97/00241. See also: Wang (1998) J. Med. Chem. 41:2207-2215, describing the synthesis of long chain alkyl acyl carnitine esters as biodegradable cationic lipids for use in gene delivery; Stegmann (1997) Biochim Biophys Acta. 1325:71-79; Liu (1996) Pharm. Res. 13:1856-1860; Templeton (1997) Nat. Biotechnol. 15:647-652. In addition, cationic lipid carriers having more than one cationic lipid species may be used to produce complexes according to the method of the present invention.

Non-Cationic Lipids

Non-cationic lipids can also be used in the complexes of the invention. For example, cationic lipid carriers can also contain a neutral lipid. If present, the neutral lipid is usually in approximately equimolar amounts with the cationic lipid. Cationic lipid and non-cationic lipids can, however, also be used in various proportions other than equimolar amounts. The neutral lipid is helpful in maintaining a stable lipid bilayer in liposomes, and can significantly affect transfection efficiency.

Usually, the cationic lipid and non-cationic lipid will be prepared as liposomes by methods known in the art. Generally, the lipids are dried to a film and resuspended in an aqueous solution. The resulting liposomes may be further reduced in size by sonication or extrusion through a membrane of fixed pore size. In one embodiment, the liposomes are rehydrated in 5% dextrose in water and heated to 50°C for 6 hours. They are then extruded through a filter.

Another useful neutral lipid is cholesterol, see, *e.g.*, Liu (1997) Nat. Biotech. (15):167-173. The effect of cholesterol on liposomes *in vivo* is described in Semple (1996) Biochem. 35:2521-2525. Other useful neutral lipids include, *e.g.*, DLPE and DiPPE, see, *e.g.*, U.S. Patent Application Serial No. (USSN) 09/054,769.

The role of non-cationic, helper lipids in cationic lipid-mediated gene delivery is described, *e.g.*, in Felgner (1994) J. Biol. Chem. 269:2550-2561, describing improved transfection using DOPE; Hui (1996) Biophys. J. 71: 590-599.

Other Complex Substituents

The lipid-nucleic acid complexes of the invention can include other compositions, such as polysaccharides or peptides or proteins, *e.g.*, to enhance cellular uptake, endosomal release or nuclear transport. Examples include, *e.g.*, polyamines, carbohydrates, synthetic polycationic polymers, polylysine, polyarginine, protamine, polybrene, histone, cationic dendrimer, and synthetic polypeptides. See, *e.g.*, WO 96/22765. See, *e.g.*, Sugimoto, et al., U.S. Patent No. 5,759,572, describing liposomes with oligosaccharides on the surface.

Synthesis of Lipid-Nucleic Acid Complexes

Typically, complexes are prepared by adding one solution to the other, *i.e.* nucleic acid to the cationic liposomes, or cationic liposomes to nucleic acid, with constant stirring. For *in vivo* uses, it is desirable to prevent the formation of macroaggregates or precipitation during the complexation process. Thus, for complexes having a net positive charge, the nucleic acid is added to the liposome suspension; for complexes having a net negative charge, the liposomes are added to the nucleic acid solution.

The liposomes are typically prepared in low ionic strength solutions, such as 5% dextrose in water. The nucleic acid is also typically prepared in a low ionic strength solution to prevent interference by additional ions with the lipid complexation process. A low-ionic strength solution means a solution having a conductivity less than about 35 mS, preferably less than about 10 mS, and most preferably less than about 1 mS. Desirably, the DNA solution will contain no salts. Typically, the DNA is in a low ionic strength solution, such as about 5% dextrose in 5 mM Tris-HCl (pH 8.0).

The nucleic acid-cationic lipid complexes of the invention can also be prepared using a reduced-volume, dual feed stream process. It involves the collision of